BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 379–387 (1998)
ARTICLE NO. RC989425

Cathepsin B-Mediated Activation of the Proinflammatory Caspase-11

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Received August 31, 1998

Members of the caspase (CASP) family of cysteine proteases can be subdivided in proapoptotic caspases and proinflammatory caspases. Whereas the apical activation pathways for the caspases that are involved in the execution of the apoptotic process are beginning to be understood, the pathways that lead to the activation of proinflammatory caspases are still largely unknown. Analysis of subcellular fractions for their ability to process and activate several caspases *in vitro* led to the identification of lysosomes as the source for a protease that could proteolytically activate the proinflammatory CASP-11. Although this lysosomal activity was sensitive to caspase inhibitors, affinity purification with the biotinylated broad spectrum caspase inhibitor z-VAD.fmk revealed the CASP-11 activating protease as cathepsin B. Activation of CASP-11 by cathepsin B as well as its sensitivity to several caspase inhibitors was further confirmed with purified proteases. Similar to the role of mitochondrial factors in the activation of proapoptotic caspases, our results suggest a potential role for lysosomes and cathepsin B as activators of specific proinflammatory caspases. In addition, the aspecific inhibition of cathepsin B by so-called specific caspase inhibitors implicates that results obtained with these inhibitors should be interpreted with care.

Caspases (CASPs), an emerging group of aspartic acid-specific cysteine proteases, seem to have a dual function in the development of multicellular organisms. Most of the currently known CASPs are involved in the process of programmed cell death or apoptosis (1). Others such as CASP-1 , CASP-4 (=murine CASP-11), and possibly CASP-5 and CASP-12 function as proinflammatory caspases, leading to the maturation of proinflammatory cytokines. CASP-1 or CASP-11 deficient mice are defective in production of mature IL-1β and resistant to endotoxic shock (2–4), which further suggests an important role of CASP-1 and –11 in inflammatory disorders. All caspases are synthesized as proenzymes with a tripartite structure consisting of an N-terminal prodomain which is variable in length, a large subunit with an approximate size of 20 kDa (p20) and a second smaller subunit of about 10 kDa (p10); the mature caspase is a $(p20p10)_2$ hetero tetramer. Processing of caspases to a mature product occurs following different apoptotic stimuli (5), and requires cleavage at specific aspartic acid residues located between the different subdomains. So far, only two types of proteases have been shown to be able of performing this type of aspartic acid specific maturation: the caspases themselves and the serine protease granzyme B (6–8). Several lines of evidence strongly suggest the existence of a procaspase activation cascade, in which

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Abbreviations used: Ac-YVAD.cmk, acetyl-Tyr-Val-Ala-Asp(Ome)-cmk; AFC, 7-amino-4-trimethyl coumarin; AMC, 7-amino-4-methyl coumarin; Apaf, apoptotic protease activating factor; CASP, caspase; CFS, cell free system; cmk, chloromethyl ketone; EST, expressed sequence tag; fmk, fluoromethyl ketone; HM, heavy membrane fraction; IL-1;gb:interleukin-1;gb; LPS, lipopolysacharide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulphonyl_fluoride; TNF, tumor necrosis factor; TNF-R, TNF receptor; TPCK, N-;ga-tosyl-L-phenylalanine chloromethyl ketone; z-AAD.cmk, benzyloxycarbonyl-ala-Ala-Asp(Ome)-cmk; z-ARR.AFC, benzyloxycarbonyl-Ala-Arg-Arg-AFC; Ac-DEVD.AMC, acetyl-Asp-Glu-Val-Asp-AMC; z-FA.fmk, benzyloxycarbonyl-Phe-Ala-fmk; z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fmk.

0006-291X/98 $25.00

the initial activation of one caspase can lead to the activation of multiple other family members. The question how an apoptotic stimulus is linked to the activation of caspases, has been partially answered by the isolation of FLICE/MACH (9, 10), later renamed as CASP-8. This protein associates through its prodomain with FADD/MORT-1 which is a component of the tumor necrosis factor-receptor 1 (TNF-R1) and FAS/APO-1 receptor complex. In the case of Fas, stimulus-induced recruitment of CASP-8 to the receptor leads to an oligomerization-induced autoprocessing and activation of CASP-8 (10, 11), which then processes and activates CASP-3 and CASP-7. These play a crucial role in the execution of apoptosis by cleaving several substrates. Recently, an alternative pathway for caspase activation that does not involve the recruitment of CASP-8 to FAS/APO-1 has been described (12). In this model cell death is triggered by the release of cytochrome c from the intermembrane space of mitochondria into the cytosol. In the presence of ATP, cytochrome c binds with the adaptor protein Apaf-1, allowing the recruitment and activation of CASP-9. The latter caspase then activates final execution caspases such as CASP-3, CASP-6, and CASP-7. A second mitochondrial component reported to be able to activate caspases after release from mitochondria is AIF (13), whose identity is still under investigation. Finally, CASP-3 and CASP-7 might also be directly activated by granzyme B or cathepsin G (7, 8, 14). In contrast to the above described activation of effector caspases that are involved in the execution of the apoptotic process, the apical activation pathways for the proinflammatory CASP-1, CASP-11 (the murine homologue of human CASP-4) and CASP-12 are not yet known. Compared to the proapoptotic caspases, the above mentioned proinflammatory caspases are very poor substrates for other caspases, although CASP-8 might also represent an efficient apical activation mechanism of CASP-11 (15, Van de Craen *et al.*, in preparation).

The purpose of the present study was to identify potential subcellular localized activators of proinflammatory caspases which may be released upon proinflammatory stimuli. We used cell fractionation and protein purification techniques to identify the lysosomal cysteine protease cathepsin B as a CASP-11 activating enzyme *in vitro*.

MATERIALS AND METHODS

Inhibitors. TPCK was obtained from Sigma Chemical Co. (St. Louis, MO). z-AAD.cmk, z-FA.fmk and Ac-YVAD.cmk were purchased from Calbiochem-Novabiochem International (San Diego, CA). z-DEVD.fmk and z-VAD.fmk were supplied by Enzyme Systems Products (Dublin, CA) and leupeptin was from Boehringer-Mannheim.

Plasmids. The cloning of cDNA of murine procaspases and their subcloning into pGEM11zf(+) (Promega Biotec, Madison, WI) has been described previously (16). The procaspase cDNA clones were used as a template for PCR amplification with Vent DNA polymerase (New England Biolabs, Beverly, MA) to prepare bacterial expression vectors for the caspases. First, a ΔproCASP-1 construct was made using synthetic oligonucleotide primers designed to eliminate the N-terminal region corresponding to the prodomain of CASP-1, to add an EcoRV restriction site upstream of an inserted methionine start codon, and to append a 10 amino acid (N)SAWRHPQFGG(C) Strep-tag to the C-terminus (17), followed by two stop codons as well as BamHI, MunI, EcoRI and XbaI restriction sites. The sense primer used was 5′-CGCGATATCGGTACCTGCCACCATGGGCACAT-TTCCAGGACTGACTGG-3′. The antisense primer, including the Strep tag, was 5′-CGCGTCTAGAGAATTCAATTGCCGGATCCT-ATTAACCACCAAACTGGGGGTGGCGCC AAGCGCTAGCA-TGTCCCGGGAAGAGGTAGAAACGT-3′. The resulting PCR product was digested with EcoRV and EcoRI, and was inserted into an EheI/EcoRI-opened pLT10TH plasmid (18), downstream of the His_6-tag to generate pLTΔproCASP-1a. This plasmid was further used as a basic vector to subclone other caspases. The sense primers 5′-CGGGGTACCTATGGCTGAAAACAAACACCCTGACA-3′ and 5′-CGGGGTACCTGCCACCATGGCACCAGGCAGCCACCATGG-TGAAGC-3′ were used to amplify full length CASP-11 and CASP-11 without a prodomain (ΔproCASP-11), respectively. The antisense primer 5′-CGCGCTAGCGTTGCCAGGAAAGAGGTAGAAATA-3′ was used for both CASP-11 amplifications. The resulting products were NcoI/NheI-cloned in pLT10TH by exchanging ΔproCASP-1 in pLT-ΔproCASP-1a with CASP-11 and ΔproCASP-11. pLT-CASP-11, containing full length CASP-11, was only used for *in vitro* transcription/translation by T7 DNA polymerase (Promega Biotec). pLT-ΔproCASP-11 was also used for bacterial expression and protein purification. The primers 5′-CGGGGTACCTGCCACCATGGC-ATCTGGGATCTATCTGGACAGTAG-3′ and 5′-CGCGCTAGC-GTGATAAAAGTACAGTTCTTTCGT-3′ were used for ΔproCASP-3 amplification. The resulting product was KpnI/NheI-cloned in pLT10TH to generate pLT-ΔproCASP-3. The forward primer 5′-GCGGATATCGATGGAGAACAACAAAACCTCAGTG-3′ and the reverse primer 5′-GCGGGATCCCTAGTGATAAAAGTACAGTTC-TTTCG-3′ were used for amplification of full length CASP-3. The resulting PCR product was digested with EcoRV/BamHI, filled in with T_4 DNA polymerase and ligated into EheI-opened pLT10TH.

Expression and purification of His-tagged ΔproCASP-11. Exponentially growing MC1061 bacteria containing the pICA2 plasmid (Mertens *et al.*, manuscript in preparation) were transformed with the pLT-ΔproCASP-11 expression plasmid and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 16 h at 20°C. The cells were harvested by centrifugation, resuspended in buffer A (20 mM Tris–HCl pH 7.5, 1 mM oxidized glutathione, 10% glycerol, 0.2 M NaCl, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml PMSF) and lysed by passage through a French press. The lysate was cleared by centrifugation at 20,000 x g for 10 min, and bacterial DNA was removed over a DEAE-Sepharose column (XK26/20; Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer A without leupeptin, aprotinin and PMSF. The flow through was subsequently loaded on a cobalt-affinity column (Talon, Clontech Laboratories, Palo Alto, CA). The column was washed overnight with buffer A, followed by removal of low strength metal binding proteins by short washing with buffer A supplemented with 10 mM imidazole. The His_6-tagged protein was eluted with 20 mM Tris–HCl pH 7.5, 1 mM oxidized glutathione, 10% glycerol, 50 mM NaCl and 100 mM imidazole. The eluate was 4-fold diluted in buffer A without NaCl, and further purified by FPLC on a Mono-Q HR 5/5 column (Pharmacia Biotech) at a flow rate of 0.5 ml/min and a linear NaCl concentration gradient. This purification protocol resulted in a clear separation of unprocessed, inactive ΔproCASP-11 from both the p20 and p10 subunits, which eluted together in the form of the active enzyme. A sample containing only unprocessed His_6-tagged ΔproCASP-11 was chosen for activation studies.

Preparation of a heavy membrane fraction and subfractionation. Livers were obtained from female C57/Bl6 mice (8–10 weeks old), cut into small cubes, and washed 4x with PBS at 4°C. The liver fragments were further homogenized with a Wheaton type B Douncer in homogenization buffer (5 mM KH_2PO_4 pH 7.4, 0.3 M sucrose, 1 mM EGTA, 5 mM MOPS). The lysate was cleared by centrifugation (up to 4-fold) at 1,800 x g for 10 min. The heavy membrane fraction (further referred as HM) was spun down at 10,000x g for 10 min and used as such, or further subfractionated using a modified Sims method (19). The HM prepared from 2 g liver was dispersed in 2 ml of 15% Percoll solution which was placed on top of three layers of 3 ml Percoll solution, 23, 40, and 60%, respectively. All Percoll solutions contained 0.3 M sucrose, 5 mM MOPS, 1 mM EGTA and 5 mM Tris–HCl (pH 7.4). The column was spun down at 31,500 x g for 5 min. The lysosomal fraction was located at the bottom of the 60% Percoll solution; the peroxisomal fraction was layered between the 60% and the 40% Percoll solutions, and the mitochondria were located between the 40% and the 23% Percoll solutions. The specific fractions were washed twice with homogenisation buffer (8x volume) and dissolved in 300 μl cell free system (CFS) buffer (10 mM Hepes-NaOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF and 1 mM dithiothreitol) supplemented with 1% NP40 for material originally derived from 1 g liver. After rotating at 4°C for 15 min the unsoluble material was spun down at 23,000x g and removed.

Determination of CASP processing. [^{35}S]Methionine-labelled caspases were prepared with an *in vitro* coupled transcription/translation TNT kit from Promega Biotec, using SP6 or T7 RNA polymerase to transcribe genes cloned in pGEM11zf(+) and pLT10TH, respectively. The reaction products were analysed by SDS–PAGE and stored at -70°C until needed. 2 μl of material was incubated for 1 h at 30°C with 20 μl of the HM, or 2 μl of the specific subfractions, or 60 ng of purified cathepsin B, D or H (Calbiochem-Novabiochem International, San Diego, CA) in a total volume of 25 μl CFS buffer, and analysed by SDS–PAGE and fluorography. In the case of peptide inhibitor studies, 20 μl of the HM was preincubated for 5 min at 30°C with synthetic inhibitors.

Affinity labelling and purification with biotin-VAD.fmk. Labeling of proteins with biotin-VAD.fmk was done by incubating 20 μl of specific cellular subfractions with 1 μM biotin-VAD.fmk for 20 min at 30°C. Proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes which were then incubated with streptavidin-conjugated horse-radish peroxidase (1/500). The affinity-labelled proteins were visualized by ECL (Amersham Life Science, Amersham, UK).

For purification purposes, a lysosomal preparation prepared from 5 rat livers was solubilised in 2 ml CFS/1% NP40 buffer. The insoluble material was spun down for 30 min at 23,000 x g and the supernatant was incubated for 20 min at 30°C with 5 μM biotin-VAD.fmk (Enzyme Systems Products, Dublin, CA). 100 μl streptavidin coupled agarose (Sigma) was added and rotated at room temperature for 20 min. The streptavidin coupled agarose was then washed 5× with 0.1 M glycine, 1% SDS, pH 6.8 and boiled in 100 μl 2× sample buffer for 10 min. Proteins were separated by 15% SDS–PAGE and either electroblotted onto Problot membranes or *in-gel* digested with trypsin (20).

N-terminal sequencing was performed according to the manufacturer's instructions using a 477A protein sequenator (Applied Biosystems Inc.). Peptides liberated by *in-gel* digestion were loaded onto a C18 RP-HPLC column (2.1 mm × 250 mm, Vydae Separations Graap, Hesperia, CA) and eluted with a linear gradient of acetonitrile in 0.1% TFA. The eluate was split: 80% into a UV absorbance detector and 20% into a Fisons VG Platform (Manchester, UK) mass spectrometer. Peptides adsorbing at 214 nm were collected, and those corresponding to single peptide masses were selected for automated Edman degradation.

Enzymatic activity assays. Catalase activity was assayed by incubating 50 μl of each subcellular fraction for 30 min at room temperature with 100 μl 0.06% H_2O_2. 20 μl of this mixture was then incubated for 5 min at room temperature with 150 μl 2,2′-Azino-di-[3-ethylbenzthiazolinesulfonate(6)] diammonium solution (5 mg/ml) and horse-radish peroxidase (1/4000, Amersham Life Science, Amersham, UK) in 0.05 M citric acid, pH 4. Absorbance was measured at 405 nm.

Hexosaminidase activity was assayed by overnight incubation of 10 μl of each subcellular fraction with 60 μl substrate solution (7.5 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide, 0.5% Triton-X-100, 0.1 mM citric acid, pH 5) at 37°C. After addition of 90 μl stop solution (10 mM EDTA, 100 mM glycine, pH 10.5) the absorbance was measured at 405 nm.

Caspase activity was measured by incubation of purified CASP-11 or cell extracts for 1 h at 30°C with 50 μM of the fluorogenic substrate Ac-DEVD.AMC (Peptide Institute, Osaka, Japan) in CFS buffer. Cathepsin B activity was measured by incubation of purified cathepsin or cell extracts for 1 h at 37°C with 50 μM of the fluorogenic substrate z-ARR.AFC (Enzyme Systems Products, Dublin, CA) in CFS buffer. The release of 7-amino-4-methyl coumarin (AMC) was continuously monitored during 60 min in a fluorometer (CytoFluor; PerSeptive Biosystems, Cambridge, MA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Free 7-amino-4-trimethyl coumarin (AFC) was monitored at an excitation wavelength of 409 nm and an emission wavelength of 505 nm. Data are expressed as increase in fluorescence as a function of time (ΔF/min).

Western blot analysis. Subcellular fractions were washed with CFS, and lysed in 10 mM Tris/HCl pH 7, 1% NP40, 200 mM NaCl, 5 mM EDTA, 1 mM PMSF. The lysates were cleared by centrifugation and protein samples (100 μg) were subjected to SDS–PAGE and transferred to Hybond nitrocellulose membranes (Amersham Life Science, Amersham, UK). These were blocked with 5% dry milk in PBS containing 0.1% Tween 20, and probed with primary and secondary antibodies as described by the manufacturer's protocol. Immunoreactivity was revealed with the enhanced chemiluminescence method (ECL, Amersham Life Science, Amersham, UK). The cytochrome c antibody (clone 7H8.2C12) was from Pharmingen (San Diego, CA). The rat cathepsin B antibody was from Upstate Biotechnology.

RESULTS AND DISCUSSION

Previous reports have shown that heavy membrane fractions enriched in mitochondria are able to induce nuclear apoptosis and activation of apoptotic effector caspases (CASP-3, -7) in a cell-free system in the presence of a cytosolic factor (21). The components involved were recently identified as cytochrome c and Apaf 1 (12). We now used a similar system to identify potential activators of proinflammatory caspases (CASP-1, -11, -12). Therefore, we tested whether [^{35}S]methionine labelled, His_6-tagged CASP-1, -11, -12, as well as the proapoptotic CASP-2, -3 and -8, were processed after incubation with a heavy membrane fraction (further referred as HM) prepared from mouse liver. Only CASP-11 and CASP-3 were found to be significantly processed, releasing a fragment of 12 kDa and 2 kDa, respectively (Fig. 1). To investigate whether the cleavage products resulted from processing at the N- or

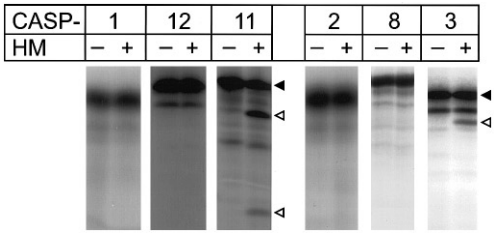

FIG. 1. Cleavage of caspases by heavy membrane fraction associated proteases [^{35}S]methionine labelled proinflamatory caspases (CASP-1, -11, -12) or proapoptotic caspases (CASP-2, -3, -8) were incubated with or without extracts from a heavy membrane fraction (HM) prepared from mouse liver and analysed by 15% SDS–PAGE. Processing products are indicated by an open arrowhead, and were only observed for CASP-3 and CASP-11. The other faster migrating protein bands are due to internal initiation of translation.

C-terminus of CASP-11 and CASP-3, we also analysed the effect of HM on CASP-3 and CASP-11 without their prodomain (ΔproCASP-11 and ΔproCASP-3). In contrast to proCASP-3, incubation of ΔproCASP-3 with HM led no longer to the removal of a 2 kDa fragment (Fig. 2A). Therefore, the 2 kDa fragment is likely to correspond to the short prodomain of CASP-3, whose removal is not sufficient for activation (5, our own unpublished results). ΔproCASP-11 was again processed with the generation of a 12 kDa band (Fig. 2B). This cleavage pattern resembles the one that is obtained after proteolytic activation of proCASP-11 by recombinant CASP-8 (15, Van de Craen *et al.*, in preparation) and in tissues of lipopolysaccharide (LPS)-treated mice (22). Consequently, the obtained *in vitro* cleavage of CASP-11 upon incubation with HM is likely to correspond to the removal of the C-terminal p10 subunit.

Activation of a procaspase implicates proteolytic processing between its p20 and p10 subunits (5). Although the prodomain can be essential to initiate dimerization (23), its removal is not a prerequisite to form active enzyme (24). We therefore analysed whether the removal of the C-terminal p10 of CASP-11 upon incubation with HM led to an increased caspase activity. Therefore, N-terminal His_6-tagged ΔproCASP-11 was expressed in *E. coli* and purified to homogeneity over a Co^{2+}-metal chelate column. Mature CASP-11 (p20/p10), which resulted from autoproteolytic processing, was separated from inactive p30 ΔproCASP-11 using a Mono-Q column. The purified inactive ΔproCASP-11 was then incubated with HM, and CASP-11 enzymatic activity was measured with the fluorogenic tetrapep-

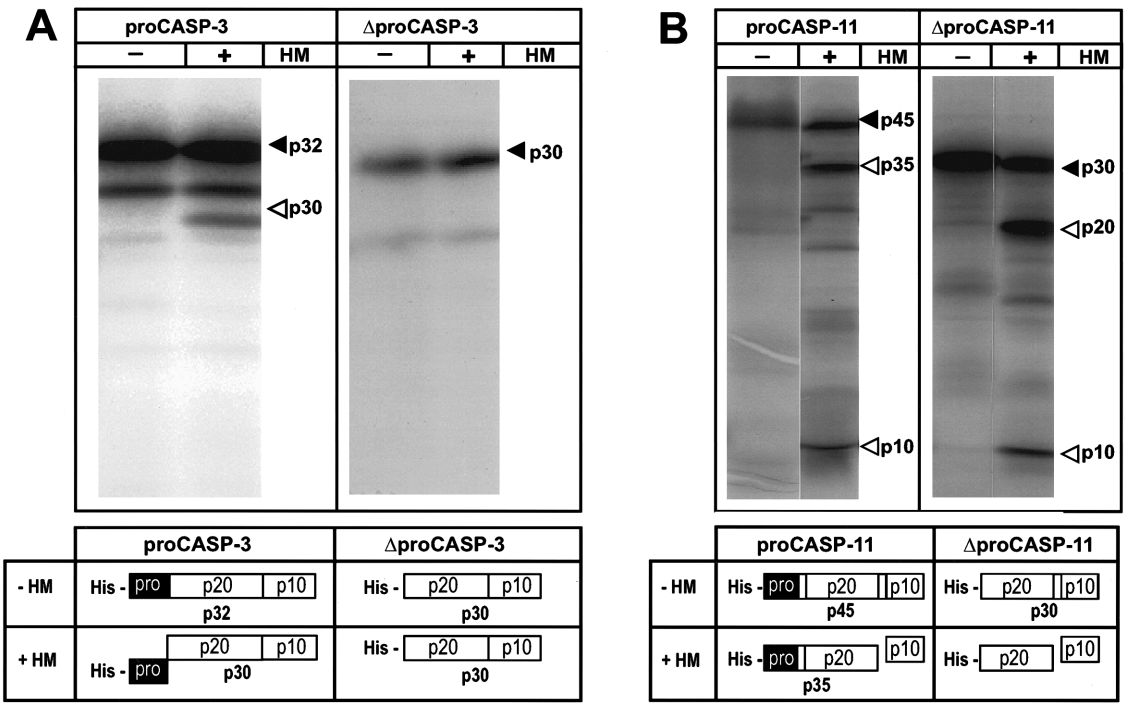

FIG. 2. CASP-3 and CASP-11 are differentially processed upon incubation with HM. Purified, [^{35}S]methionine labelled His_6-tagged proCASP-3 and ΔproCASP-3 (A) or proCASP-11 and ΔproCASP-11 (B) were incubated with or without extracts from HM prepared from mouse liver as described in Materials and Methods and analysed by 15% SDS–PAGE. Unprocessed CASP-3 and CASP-11 are indicated by a closed arrowhead, whereas processing products are indicated by an open arrowhead. Protein bands are referred according to the size of the products that are normally generated upon caspase activation as shown at the bottom. In the case of proCASP-3, the faster migrating protein band is due to internal initiation of translation.

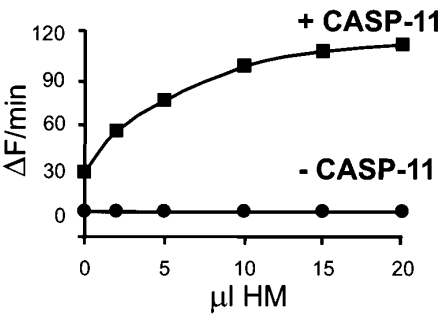

FIG. 3. Activation of CASP-11 upon incubation with HM. Four micrograms of purified unprocessed recombinant ΔproCASP-11 (■) was preincubated for 5 min at room temperature with a serial dilution of HM and subsequently analysed for its proteolytic activity on Ac-DEVD.AMC. The increase in fluorescence as a function of time (ΔF/min) was used as a parameter for the amount of AMC that was released. Incubation of Ac-DEVD.AMC with HM in the absence of CASP-11 (●) did not induce processing.

tide substrate Ac-DEVD.AMC. Figure 3 shows that ΔproCASP-11 was activated upon incubation with HM, which on its own had no detectable activity on the fluorogenic substrate. Therefore, the CASP-11 fragments generated upon incubation with HM clearly led to active CASP-11.

As caspases have been shown to function in a hierarchic order by heteroprocessing (6), we studied the effect of several protease inhibitors, including caspase inhibitors, on the HM-mediated processing of [^{35}S]methionine labelled and His-tagged ΔproCASP-11 that was purified on a Co^{2+}-metal chelate column (Fig. 4). Processing of CASP-11 was not inhibited by the chymotrypsin-like protease inhibitor TPCK (100 μM), nor by the irreversible granzyme B inhibitor z-AAD.cmk (10 μM). However, processing was completely inhibited by the irreversible caspase inhibitors z-DEVD.fmk and z-VAD.fmk (10 μM), and partially inhibited by Ac-YVAD.cmk (10 μM). These results suggest that the HM associated protease which cleaves CASP-11 corresponds to a caspase-like enzyme. However, CASP-11 processing was also completely inhibited by the cysteine protease inhibitor leupeptin (1 μM), which is not expected to inhibit caspases.

The sensitivity of the HM-associated processing of CASP-11 to caspase inhibitors as well as to the non-caspase inhibitor leupeptin, suggested that two types of proteases were involved in CASP-11 processing. To identify these proteases, we used Percoll gradient centrifugation to further subfractionate the original HM into three subfractions: mitochondria, peroxisomes and lysosomes. The purity of the fractions was verified using Western blot analysis of the mitochondrial protein cytochrome c (Fig. 5A), enzymatic assays for the specific lysosomal protein hexosaminidase (Fig. 5B) and the peroxisomal protein catalase (Fig. 5C), as well as electron microscopy (data not shown). When these subfractions were analysed for processing of [^{35}S]methionine labelled and His_6-tagged ΔproCASP-11, it was found that activity was highly enriched in the lysosomal fraction (Fig. 5D). The latter was rather surprising, especially in view of the above described sensitivity of CASP-11 processing to caspase inhibitors such as z-VAD.fmk. Therefore, we used the biotinylated irreversible caspase inhibitor z-VAD.fmk as affinity label to isolate the lysosomal protease responsible for CASP-11 processing. This approach has previously been used to visualise active caspases on a Western blot with streptavidin-conjugated peroxidase (25, 26). Control experiments with purified CASP-1 revealed that biotin-VAD.fmk covalently labelled the p20 subunit of CASP-1 (Fig. 6A). When this reagent was incubated with lysosomal extracts from rat liver cells and analysed on a Western blot, four discrete bands were affinity labelled: HMP_1 (34 kDa), HMP_2 (32 kDa), HMP_3 (28 kDa) and HMP_4 (25 kDa, Fig. 6A). In contrast, no protein labeling was detectable in the other fractions, except for one protein in the peroxisomal fraction which likely results from a contamination with lysosomal proteins. To identify the labelled proteins, a similar affinity labeling was performed on a lysosomal preparation of rat liver cells, and biotin-VAD.fmk labelled proteins were purified on a streptavidin coupled agarose matrix as described under Materials and Methods. Proteins were separated by SDS–PAGE, elec-

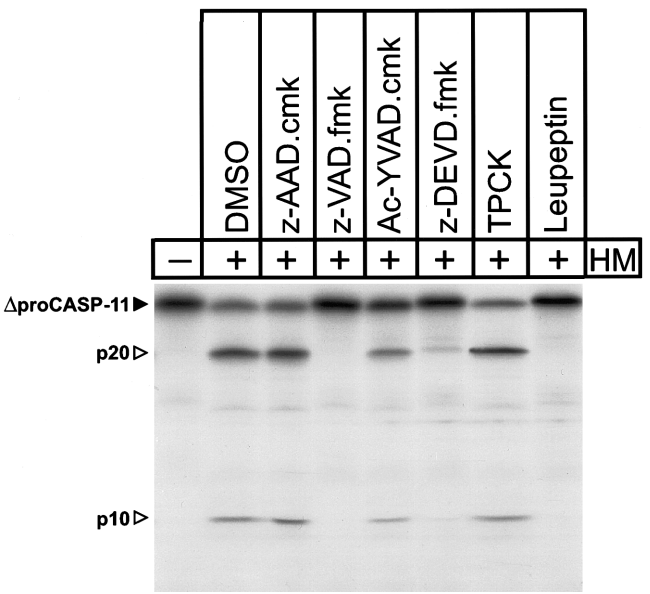

FIG. 4. Protease inhibitor sensitivity of HM-induced CASP-11 processing. Extracts from mouse liver HM were preincubated with the indicated inhibitors for 5 min at 30°C and subsequently incubated for 1 h at 30°C with purified [^{35}S]methionine labelled ΔproCASP-11. DMSO, a solvent for all inhibitors, was used as a negative control. Processing was analysed by 15% SDS–PAGE. Unprocessed CASP-11 and processing products are indicated by closed and open arrowheads, respectively.

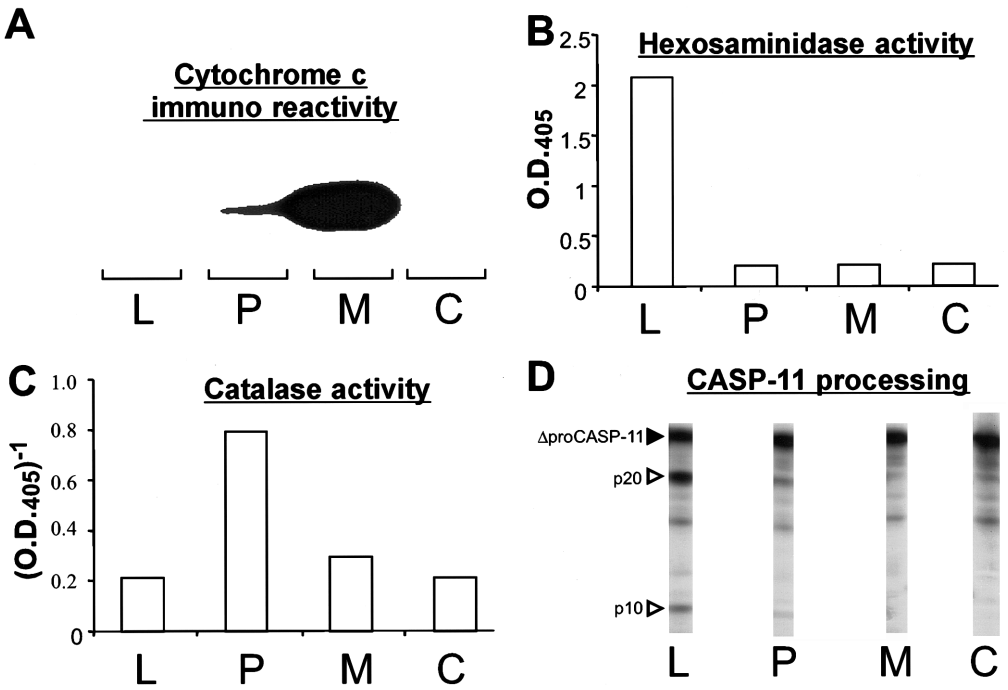

FIG. 5. Subfractionation of HM reveals lysosomes as the source of CASP-11 processing activity. HM prepared from mouse liver was subfractionated into lysosomes (L), peroxisomes (P) and mitochondria (M) on a discontinuous Percoll gradient. Cytosol (C) was used as a control. Purity of the fractions was verified by analysing the expression or activity of specific marker proteins: (A) Western blotting with anti-cytochrome c (mitochondria); (B) hexosaminidase activity (lysosomes); (C) catalase activity (peroxisomes). (D) Extracts from the different subfractions were incubated with purified [^{35}S]methionine labelled ΔproCASP-11 and processing was analysed by 15% SDS–PAGE. Unprocessed CASP-11 and processing products are indicated by closed and open arrowheads, respectively.

troblotted onto Problot-membranes as described previously (20), and subjected to N-terminal microsequencing. Band HMP_2 and HMP_4 revealed N-terminal sequences which matched perfectly with amino acids residues 80-89 and 129-137 of the light and heavy chain of rat cathepsin B, respectively (Fig. 6B). Similarly, the N-terminal sequence of HMP_3 showed 90% identity to residues 114-123 of the heavy chain of rat cathepsin H. Microsequencing of HMP_1 failed to reveal a clear result and therefore we made a second purification run and performed *in-gel* digestion on the HMP_1 band. The resulting tryptic peptides were then separated on a reverse-phase HPLC column, and the sequence of two peptides was determined (NSWGEPWGER and VGDYGSLSGR). A BLAST search with these peptides revealed no corresponding protein. However, both sequences showed perfect homology to the human EST clone 182180, which was described as a new cathepsin-like protease. A polyclonal antibody against human cathepsin B was cross-reactive with HMP_2 and HMP_4 (data not shown), confirming the results of the N-terminal sequencing. The above finding that all proteins that were affinity labelled with biotin-VAD.fmk correspond to cysteine proteases that belong to the cathepsin family was rather surprising, because z-VAD.fmk is widely used as a specific inhibitor of caspases. Therefore, purified human cathepsin B, -H and -D were tested for their ability to cleave [^{35}S]methionine labelled CASP-11 in the absence or presence of z-VAD.fmk. Only cathepsin B could process CASP-11 to its p20 and p10 subunits (Fig. 7). Moreover, cathepsin B activity was sensitive to z-VAD.fmk, which was 50% effective at a concentration of 200 nM. A similar inhibition was observed with z-DEVD.fmk and Ac-YVAD.cmk (data not shown). Inhibition of apoptosis by these inhibitors has been used in numerous studies as a useful tool to determine the involvement of caspases (28 and references cited herein). In such studies, cells are mostly incubated with the caspase inhibitors at concentrations of 10–50 μM. In our study, we clearly show that such concentrations also non-specifically inhibit purified cathepsin B. Moreover, incubation of cells with 10 μM z-VAD.fmk completely inhibited cathepsin B activity in cellular extracts (unpublished observations). The reason for this non-specific inhibition is still unclear. Peptidylfluoromethane and peptidylchloromethane inhibitors are since long used as inhibitors of cysteine proteases by irreversibly alkylating a cysteine residue in the active site of the protease (29). A well-known disadvantage of such inhibitors is the reactivity of the fmk- or cmk-group towards thiol groups in non-proteases such as

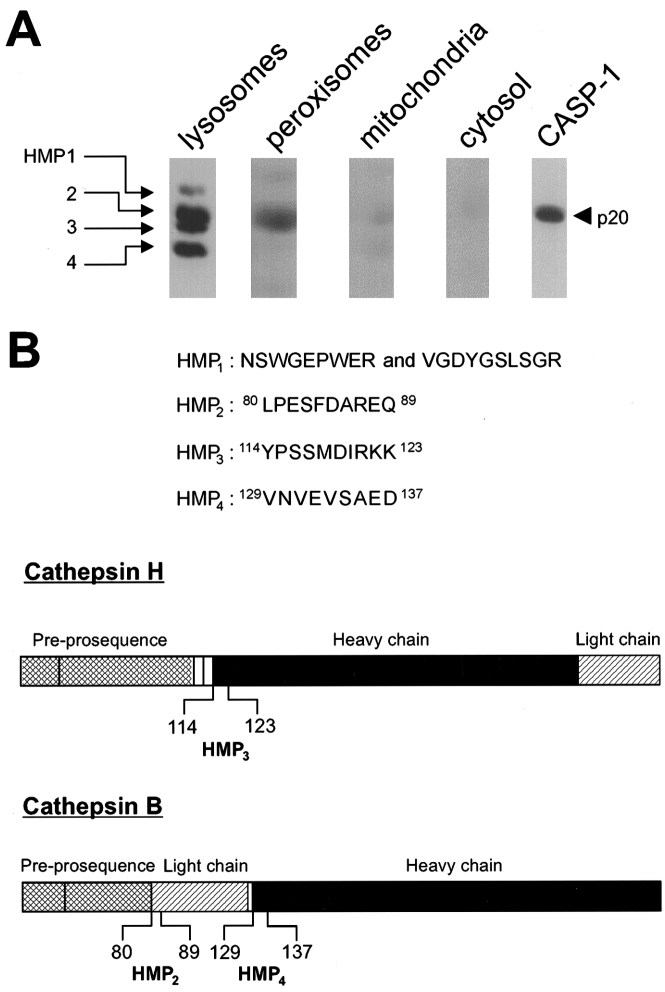

FIG. 6. (A) Affinity labelling with biotin-VAD.fmk. 100 ng of purified recombinant CASP-1 or aliquots containing 20 μl of the indicated subcellular fractions prepared from rat liver cells were incubated for 20 min at 30°C with 1 μM biotin-VAD.fmk. Proteins were separated by SDS–PAGE and affinity labelled proteins (HMP_{1-4}) were revealed as described in Materials and Methods. In the case of CASP-1 the labelled polypeptide corresponds to the p20 subunit. (B) N-terminal sequencing of HMP_{1-4}. Lysosomal extracts from rat liver cells were incubated with 5 μM biotin-VAD.fmk. Affinity labelled proteins were purified on a streptavidin-coupled agarose matrix, separated by 15% SDS–PAGE, and used for sequencing. N-terminal sequences (HMP_{2-3}) or tryptic peptide sequences (HMP_1) are shown, as well as their localisation in the corresponding cathepsins.

glutathione. However, the inhibition of cathepsin B with z-VAD.fmk was not solely mediated by the reactivity of the fmk-group as no inhibitory effect could be detected with z-AAD.fmk. It seems that the fused peptide sequence also determines the inhibitory effect for cathepsin B. Anyway, conclusions on the involvement of caspases that are based on studies with these caspase inhibitors should be interpreted with care.

What could be the function of cathepsin B-mediated processing of CASP-11? Recently, CASP-11 deficient mice were shown to be resistant to LPS-induced IL-1 production and septic shock (4). Moreover, expression of CASP-11 is highly inducible by LPS. These findings underlined the important role of CASP-11 in inflammatory responses. Cathepsin B has also been implicated in a number of inflammatory diseases and pathological conditions, such as bronchitis, rheumatoid arthritis, acute pancreatitis, and cancer progression (30). In addition, cathepsin B expression and activity has been shown to be highly inducible by LPS in the macrophage-like tumor cell line THP-1 (31). However, we were unable to detect increased cathepsin B activity in this cell line, nor in the monocyte/macrophage cell line PU5-1.8 (unpublished observations), suggesting that specific conditions might be required. Preliminary experiments also did not reveal a protective effect of the cathepsin B inhibitors E64 and CA-074 on LPS-induced activation of CASP-11 or IL-1β production in these cell lines. However, the role of cathepsin B in CASP-11 activation might be masked by its redundant activation by CASP-8 (15), or may be restricted to specific inflammatory conditions.

Since most caspases reside in the cytoplasm, cathepsins may have to cross the lysosomal membrane. Such redistribution has already been described for cathepsin D which is released into the cytosol upon oxidative stress of cardiomyocytes (32) or during ischemia (33). It should be noted that cathepsin B could process CASP-11 at acidic pH as well as at neutral pH (data not shown), which is an absolute requirement for a cytosolic mode of action. Release of cathepsins into the

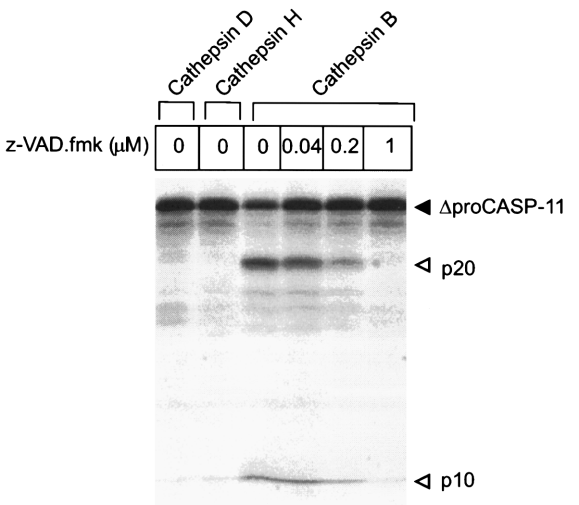

FIG. 7. Processing of CASP-11 by purified cathepsin B and its inhibition by z-VAD.fmk. Purified cathepsin B, D and H (60 ng) were preincubated for 5 min at 30°C with the indicated concentration of z-VAD.fmk followed by 1 h incubation at 37°C with [^{35}S]methionine labelled ΔproCASP-11. Processing of CASP-11 was analysed by 15% SDS–PAGE. Unprocessed ΔproCASP-11 and processing products are indicated by closed and open arrowheads, respectively.

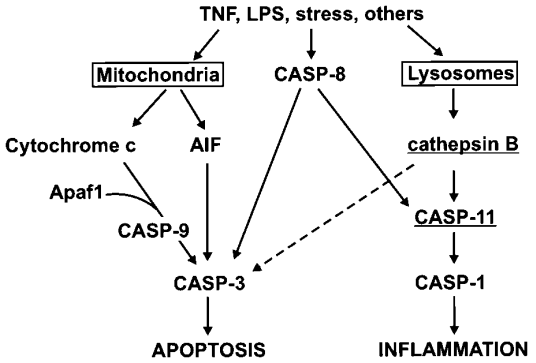

FIG. 8. Hypothetical model for the role of subcellular compartments in caspase activation. See text for details.

cytosol might be mediated by the disturbance of lysosomal membranes by an acidic sphingomyelinase which has already been implicated in signalling by several inflammatory mediators (34). Alternatively, cathepsin B might also colocalize with CASP-11 during autophagy, which is a general response of mammalian cells to serum deprivation and usually involves the sequestration of intracellular organelles and cytoplasm in autophagosomes that subsequently fuse with lysosomes (35).

In summary, we have identified CASP-11 as a novel substrate for cathepsin B, implicating a potential role for lysosomes in the initiation of caspase activation. A similar compartmentalization of caspase activators has already been demonstrated in the case of CASP-9 and CASP-3, which are activated upon the release of cytochrome c or AIF, respectively, from the mitochondria. Whereas the latter caspases are mainly involved in the execution of apoptotic processes, CASP-11 is crucial for the activation of CASP-1 and for proIL-1β maturation, implicating CASP-11 and its activation by the lysosomal protease cathepsin B in signalling pathways leading to inflammation (Fig. 8). Our study also underlines the aspecific effect of some commonly used caspase inhibitors on cathepsin B and perhaps many other proteases in the cell. Therefore, the use of such caspase inhibitors to reveal the role of caspases in biological responses should be accompanied by appropriate controls. Finally, the inhibition of a house keeping protease such as cathepsin B might also limit the therapeutic use of fmk- and cmk-derived caspase inhibitors.

ACKNOWLEDGMENTS

P.S. and G.V.L are research fellows with the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-technologisch Onderzoek in de Industrie; W.V.C. is a research assistant, and P.V. and R.B. are postdoctoral research assistants with the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen. Research was supported by the Interuniversitaire Attractiepolen, the Sportvereniging tegen Kanker, the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (Grant 9005097N), an EC Biomed 2 grant (BMH4-CT96-0300), and an EC-TMR grant (ERBFMRXCT970153). We are grateful to Marc Goethals and Magda Puype for protein sequencing and to Ilse Vandenbrande for expression and purification of CASP-11.

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